

# Posttranslational regulation of the mammalian circadian clock by cryptochrome and protein phosphatase 5

Carrie L. Partch\*, Katherine F. Shields, Carol L. Thompson†, Christopher P. Selby, and Aziz Sancar\*

Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, NC 27599

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**The molecular oscillator that drives circadian rhythmicity in mammals obtains its near 24-h periodicity from posttranslational regulation of clock proteins. Activity of the major clock kinase casein kinase I (CKI)  $\epsilon$  is regulated by inhibitory autophosphorylation. Here we show that protein phosphatase (PP) 5 regulates the kinase activity of CKI $\epsilon$ . We demonstrate that cryptochrome regulates clock protein phosphorylation by modulating the effect of PP5 on CKI $\epsilon$ . Like CKI $\epsilon$ , PP5 is expressed both in the master circadian clock in the suprachiasmatic nuclei and in peripheral tissues independent of the clock. Expression of a dominant-negative PP5 mutant reduces PER phosphorylation by CKI $\epsilon$  *in vivo*, and down-regulation of PP5 significantly reduces the amplitude of circadian cycling in cultured human fibroblasts. Collectively, these findings indicate that PP5, CKI $\epsilon$ , and cryptochrome dynamically regulate the mammalian circadian clock.**

circadian rhythm | period

Circadian rhythms organize the systemic coordination of physiological and behavioral processes of an organism with the daily solar cycle. The molecular oscillator that generates the clock consists of two interconnected transcription/translation feedback loops (1, 2). The positive arm of the major feedback loop is driven by basic helix–loop–helix–PAS (Per–Arnt–Sim) domain-containing transcription factors CLOCK and BMAL1 (3, 4). The CLOCK/BMAL1 heterodimer activates transcription of core clock genes *cryptochrome* (*Cry1* and *Cry2*), *period* (*Per1* and *Per2*), and *Rev-Erba*. PER and CRY proteins translocate to the nucleus, where they interact with CLOCK/BMAL1 to down-regulate transcription, generating the negative arm of the major feedback loop (5–7).

Posttranslational modification of clock proteins determines their subcellular localization, intermolecular interactions, and stability and is critical for establishing the 24-h periodicity of the clock (8, 9). The major clock kinase casein kinase I (CKI)  $\epsilon$  and a related kinase, CKI $\delta$ , regulate the negative arm of the major feedback loop through phosphorylation of PER proteins. The importance of PER phosphorylation is underscored by mutations in CKI $\epsilon$ / $\delta$  kinases or of phosphoacceptor sites in PER proteins that result in altered circadian periods in mammals, including humans, where deviations from normal periods manifest as sleep phase disorders (10–12). PER phosphorylation is deregulated in the absence of cryptochromes, leading to constitutive nuclear localization and/or degradation of PER proteins, suggesting that cryptochromes modulate PER phosphorylation and stability (2, 13).

The kinase activities of CKI $\epsilon$  and CKI $\delta$  are tightly regulated by inhibitory autophosphorylation, requiring dephosphorylation of up to eight sites for activation (14). Although serine/threonine phosphatases such as protein phosphatase (PP) 1, PP2A, and PP2B are capable of activating CKI $\epsilon$ / $\delta$  *in vitro*, specific physiological activators of CKI $\epsilon$  have not been identified (15). Moreover, CKI $\epsilon$ / $\delta$  do not appear to be maintained in an active state constitutively *in vivo*; the activity of CKI $\epsilon$ / $\delta$  in more than one pathway occurs only after a stimulus and may rely on different phosphatases depending on the signaling pathway or cellular context (16, 17). The role of phosphatases in the clock is therefore likely to be complex, because they

may be involved in stimulating CKI $\epsilon$ / $\delta$  activity and/or act in direct opposition to clock kinases by dephosphorylating PER proteins. In *Drosophila*, PP2A opposes the activity of DBT (the CKI $\epsilon$  homolog) to stabilize PER (18), and a similar role has been proposed for an unidentified, calyculin A-sensitive phosphatase in mammals (19). However, the *Neurospora* clock is differentially regulated by two phosphatases, homologs of PP1 and PP2A. The phosphorylation state and stability of the core clock protein FREQUENCY (FRQ) are different in strains containing mutants of these two phosphatases, although they both dephosphorylate FRQ *in vitro*, suggesting that they target FRQ differently to yield dissimilar phenotypes (20).

We previously reported that human cryptochromes 1 and 2 interact with the serine/threonine phosphatase PP5 and inhibit its activity, and we suggested that PP5 might function downstream of cryptochrome (21). Here we show that PP5 interacts with the major clock kinase CKI $\epsilon$  to regulate its activity both *in vitro* and *in vivo* and that cryptochrome inhibits PP5 noncompetitively to regulate CKI $\epsilon$  activity. Knockdown of PP5 by short hairpin RNA significantly impairs circadian cycling in cultured cells, resulting in low-amplitude oscillations of PER1 and PER2. Collectively, these data indicate an important role for PP5 in the mammalian circadian clock.

## Results

**PP5 Is Expressed in the Suprachiasmatic Nuclei (SCN) and Liver Independent of the Clock.** All clock proteins, including cryptochromes (22), are highly expressed in the master clock located in the SCN. To determine whether PP5 colocalizes with cryptochromes in the SCN, PP5 mRNA expression was analyzed by *in situ* hybridization. Although previous reports have suggested that PP5 expression is ubiquitous (23, 24), we find that PP5 mRNA is enriched in the SCN with respect to other subcortical regions of the brain (Fig. 1A). However, unlike clock genes under the transcriptional control of CLOCK/BMAL1, such as *Per1* and *Per2*, the level of PP5 mRNA did not oscillate between day [Zeitgeber time (ZT) 8] and night (ZT20) (data not shown). Similarly, when we analyzed the abundance of clock proteins in the liver, we found that expression of both PP5 and CKI $\epsilon$  was constant throughout the day (Fig. 1B). Moreover, PP5 and CKI $\epsilon$  were unaffected by the absence of both cryptochromes, which abolishes the molecular clock and increases expression of genes controlled by CLOCK/BMAL1 (Fig. 1B, last two lanes) (7, 8), indicating that both PP5 and CKI $\epsilon$  are expressed independent of the clock.

Conflict of interest statement: No conflicts declared.

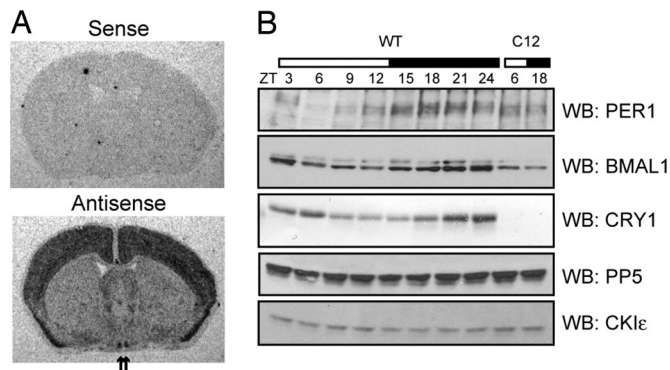
Abbreviations: CKI, casein kinase I; PP, protein phosphatase; TPR, tetratricopeptide repeat; SCN, suprachiasmatic nuclei; HA, hemagglutinin; ZT, Zeitgeber time; WB, Western blot.

\*Present address: Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75390.

†Present address: Allen Institute for Brain Science, Seattle, WA 98103.

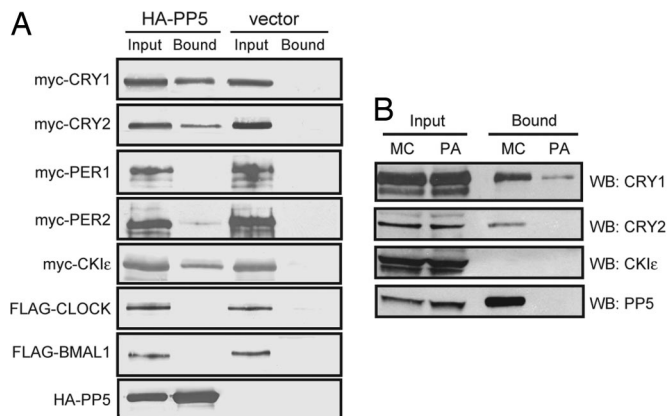
‡To whom correspondence should be addressed. E-mail: [aziz.sancar@med.unc.edu](mailto:aziz.sancar@med.unc.edu).

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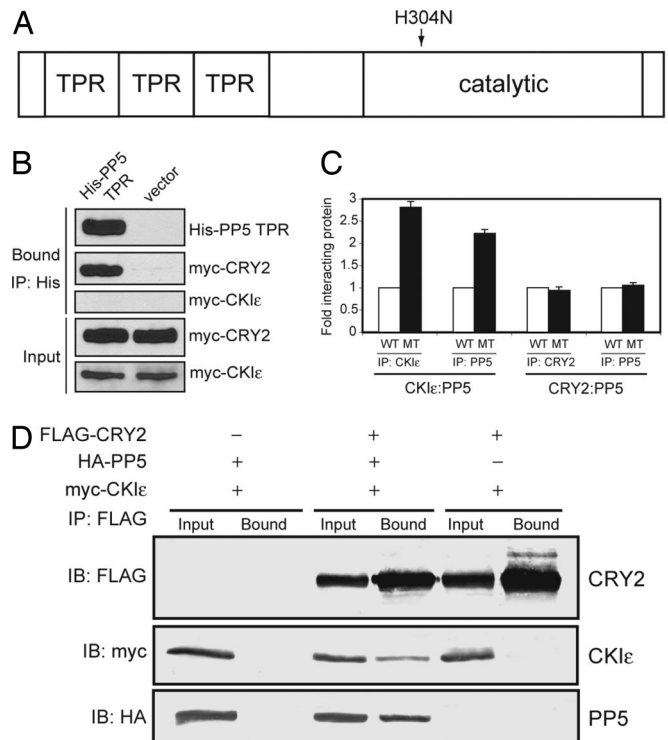


**Fig. 1.** PP5 expression is independent of the clock. (A) *In situ* hybridization was done on coronal sections of mouse brain using sense or antisense <sup>35</sup>S-labeled riboprobes for PP5. Double arrows indicate SCN. (B) Expression of core clock proteins and PP5 over circadian time in mouse liver. Total protein extract from WT and *Cry1<sup>-/-</sup>;Cry2<sup>-/-</sup>* (C12) mice killed at indicated ZTs (ZT0 = lights on) was analyzed by WB with indicated antibodies.

**Interaction of PP5 with Core Clock Proteins.** To investigate the potential role of PP5 in the clock, we examined the interaction of PP5 with core clock proteins by coimmunoprecipitation assay. HEK293T cells were transfected with hemagglutinin (HA)–PP5 and the individual clock proteins, precipitated with anti-HA antibodies, and probed for target proteins by Western blotting (WB) (Fig. 2A). In agreement with previous data (21), both CRY1 and CRY2 were precipitated by PP5. In addition, we detected an interaction between PP5 and the clock kinase CK1 $\epsilon$ . No interaction was detected between PP5 and PER1, PER2, CLOCK, or BMAL1 under our conditions. We used the rat retinal ganglion cell line RGC-5 (25), which expresses cryptochromes at relatively high levels compared with most established cell lines, to investigate interactions of PP5 with clock proteins under physiological conditions. Microcystin is a microalgal toxin that binds with picomolar affinity to the active site of the PP5 phosphatase (26, 27) and has been used to purify endogenous PP5 by affinity chromatography (28). Endogenous CRY1 and CRY2 coprecipitated with PP5 (Fig. 2B); however, we were not able to detect an interaction between microcystin-bound PP5 and CK1 $\epsilon$ . This finding may be due to one or more factors: the PP5:CK1 $\epsilon$  interaction may be more transient



**Fig. 2.** PP5 interacts with clock proteins. (A) HEK293T cells were transfected with HA-PP5 or empty vector and the indicated FLAG- or myc-tagged clock proteins, immunoprecipitated with anti-HA resin, and probed with antibodies to indicated epitope tags. (B) Interaction of PP5 with endogenous clock proteins. PP5 was precipitated from RGC-5 cell extract with microcystin-conjugated agarose (MC) or protein A (PA) agarose (negative control) and probed with indicated antibodies.



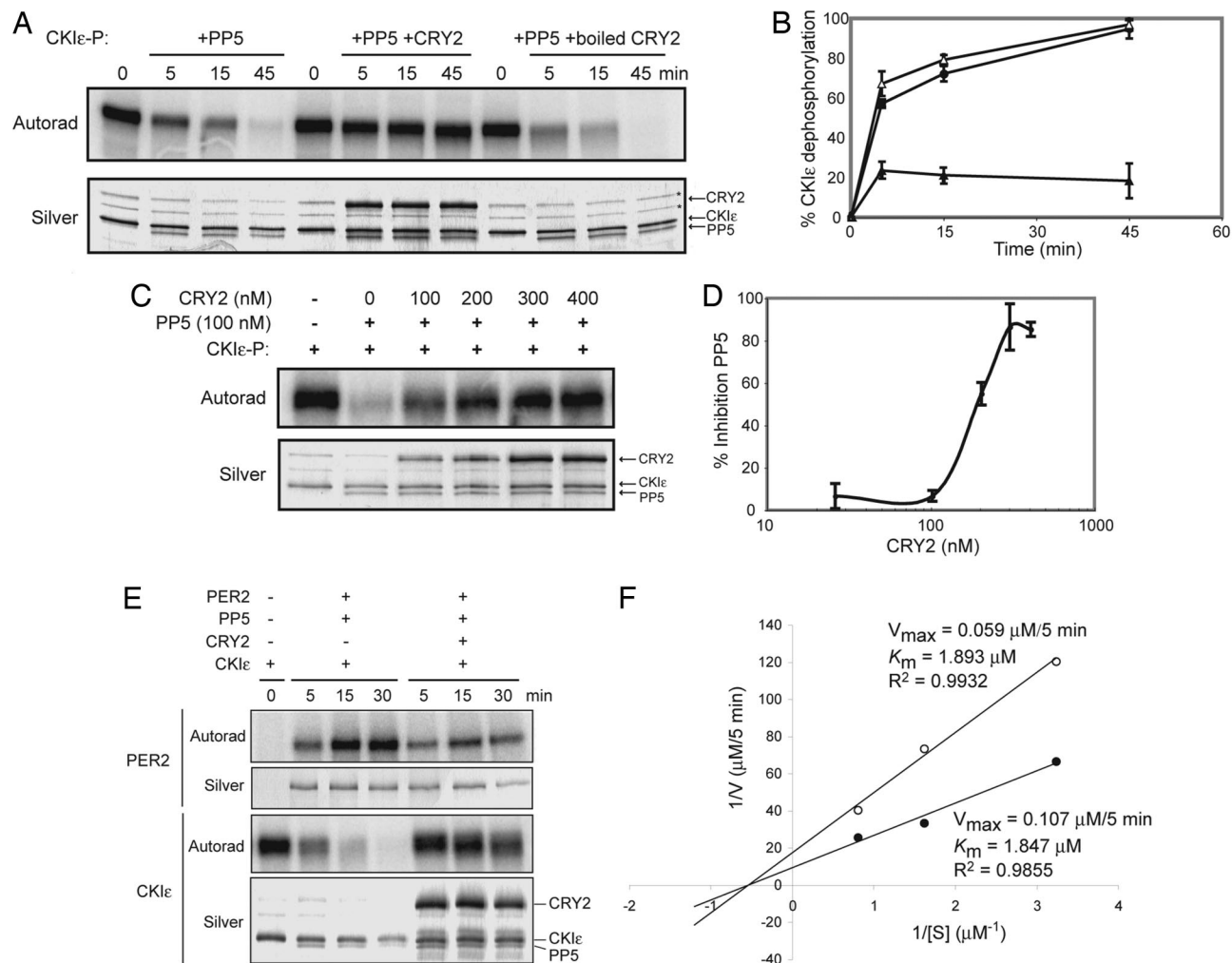
**Fig. 3.** Effect of PP5 catalytic activity on interaction with CRY2 and CK1 $\epsilon$ . (A) Schematic of PP5 protein domains. PP5 has an N-terminal autoregulatory domain with three TPR and a C-terminal catalytic serine/threonine phosphatase domain (H304 is a catalytic site residue). (B) Interaction of TPR domains with CRY2. HEK293T cells were transfected with His-PP5 TPR or empty vector and CRY2 or CK1 $\epsilon$ , immunoprecipitated with anti-His agarose, and probed with indicated antibodies. (C) Effect of PP5 catalytic activity on interaction with clock proteins. Protein coprecipitated with PP5(H304N) (MT; black) was quantified by densitometry and normalized to the amount of protein interacting with WT PP5 (white). (D) PP5 forms a ternary complex with CRY2 and CK1 $\epsilon$ . HEK293T cells were transfected with the indicated expression constructs, and CRY2 precipitates were analyzed by WB to epitope tags.

and therefore harder to capture than the PP5:CRY interaction, or microcystin binding to the active site of PP5 may preclude interaction with CKIε.

**Effect of PP5 Phosphatase Activity on Clock Protein Interactions.** PP5 contains three tetratricopeptide repeat (TPR) domains in its N terminus (Fig. 3A), which act as an autoregulatory module to inhibit phosphatase activity (29). We previously showed that CRY and PP5 interact through the TPR domains of PP5 (21), and we confirmed this finding by coimmunoprecipitation of CRY2 with His-TPR from HEK293T cells (Fig. 3B). In contrast, CKI $\epsilon$  did not interact measurably with the PP5 TPR domains. If CKI $\epsilon$  interacts with the catalytic site of PP5, disruption of PP5 catalytic activity might stabilize the PP5:CKI $\epsilon$  interaction. The PP5(H304N) mutant is catalytically inactive but maintains the normal subcellular distribution of PP5 (30). We tested for quantitative differences in protein-protein interactions by reciprocal coimmunoprecipitation of WT or mutant PP5(H304N) with CRY2 or CKI $\epsilon$  from HEK293T cells. Disruption of PP5 phosphatase activity had no effect on the PP5:CRY2 interaction but increased the stability of the PP5:CKI $\epsilon$  complex by >2-fold (Fig. 3C). Furthermore, loss of phosphatase activity did not stabilize a previously undetected complex with either PER protein, although both could interact with CKI $\epsilon$ , indicating that they were properly folded (Fig. 7, which is published as supporting information on the PNAS web site). These data collectively suggest that the TPR domain of PP5 interacts with



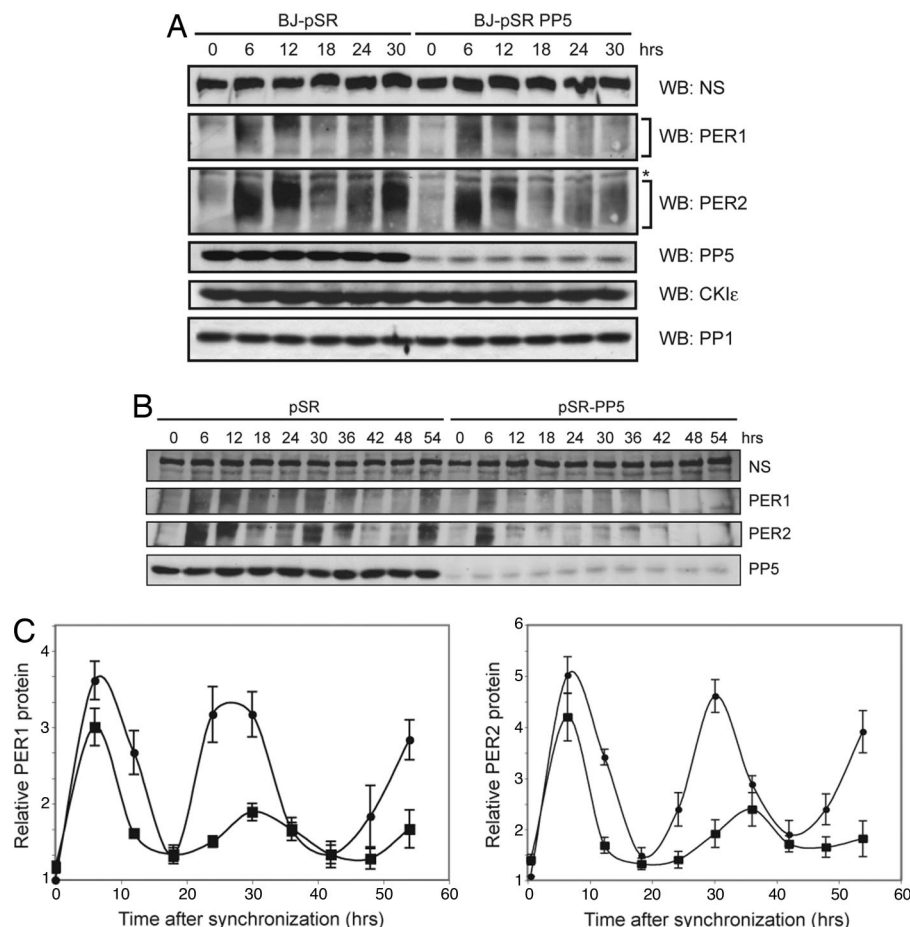




Phosphorylation of PER proteins by CKI $\epsilon$  is a critical step in execution of the negative arm of the major feedback loop; PER1 lacking CKI $\epsilon$  phosphoacceptor sites is retained in the cytoplasm, even in the presence of cryptochromes (36). Furthermore, inhibition of CKI $\epsilon/\delta$  by chemical inhibitors interferes with normal circadian cycling (9). Our data therefore suggest that PP5 may be the predominant regulator of CKI $\epsilon$  function in the molecular clock, because PP5 regulates CKI $\epsilon$  activity both *in vitro* and *in vivo* and down-regulation of PP5 compromises circadian cycling significantly in culture. We cannot rule out the possibility that down-regulation of PP5 contributes in an unidentified, additional way to disruption of clock regulation. However, the low-amplitude oscillation of hypophosphorylated PER and possible increase in period length observed upon down-regulation of PP5 are similar to the phenotypes of loss-of-function kinase mutations in *Neurospora* (37) and *Drosophila* (38) clocks, suggesting that the major role of PP5 in the clock is regulation of CKI $\epsilon$  activity. The participation of phosphatases in the mammalian clock will almost certainly expand beyond PP5, because PER proteins are also likely to be regulated by phosphatases that directly oppose the activity of CKI $\epsilon$  (18).

Mutations affecting CKI $\epsilon$  kinase activity and phosphorylation of PER proteins lead to multiple and conflicting phenotypes in *Drosophila* and mammals (10–12, 39). This phenotypic variability may result from the bimodal regulation of PER function by phosphorylation, in which phosphorylation appears to be first required for nuclear entry/retention to repress transcription, but which subsequently leads to loss of function due to degradation. We showed that down-regulation or inhibition of PP5 by dominant-negative phosphatase decreases phosphorylation of PER proteins by CKI $\epsilon$  but also results in a decrease in PER stability, suggesting a complex mode of PER regulation by CKI $\epsilon$  and PP5. Regulation of PP5 by CRY may further contribute to the bimodal regulation of PER; several studies have shown that PER proteins are constitutively phosphorylated in the absence of cryptochromes (2, 13), suggesting that cryptochromes function as a rheostat to control PER phosphorylation by CKI $\epsilon$  and PP5. The detailed mechanisms behind the bimodal regulation of PER function by phosphorylation clearly require further study.

Although expression of most clock proteins oscillates, there are notable exceptions (40). In particular, expression of the clock



**Fig. 6.** Down-regulation of PP5 disrupts circadian cycling. (A) Knockdown of endogenous PP5 decreases PER phosphorylation and impairs circadian induction of PER protein. Circadian cycling was induced in stably transfected BJ fibroblast cultures (WT control, pS<sub>R</sub>; PP5 knockdown, pS<sub>R</sub>-PP5) by serum shock, samples were harvested at the indicated times (hours after initiation of serum shock), and proteins were monitored by WB with indicated antibodies. Brackets show phosphorylated species of PER proteins. NS is a nonspecific band from the PER1 antibody, used as a loading control. An asterisk indicates a nonspecific band reacting with PER2 antibody. (B) Down-regulation of PP5 decreases the amplitude of PER protein cycling. Serum-synchronized cultures were followed for two cycles and analyzed by WB. (C) Quantitative analysis of PER protein expression in serum-induced cultures. Circles, pS<sub>R</sub> cell line; squares, pS<sub>R</sub>-PP5 cell line.

kinases and phosphatase catalytic subunits in *Neurospora* (20, 41), *Drosophila* (18, 42), and mammals (43) is constitutive, indicating that clock protein levels oscillate but the enzymes that modulate their activity and stability do not. Both PP5 and CKI $\epsilon/\delta$  have functions outside of the molecular clock, implicated in processes as diverse as cell cycle regulation and DNA damage responses, the Wnt/ $\beta$ -catenin pathway, and apoptotic signaling (44–47). Because molecular circadian rhythms are intrinsic to nearly every cell in the mammalian body, it is of considerable interest to determine whether circadian regulation of the activity of PP5 and CKI $\epsilon$  contributes to the function of these proteins in other pathways, providing molecular links between the circadian cycle and its regulation of physiological processes.

## Materials and Methods

**Plasmids.** Human CKI $\epsilon$  was cloned into pcDNA4B/myc-His from pRSETB human CKI $\epsilon$  (D. Virshup, University of Utah, Salt Lake City). *hPP5* [full-length or TPR only (residues 1–179)] was amplified by RT-PCR and cloned into pcDNA4B with an HA tag; the hPP5(H304N) mutant was generated by using the QuikChange Kit (Stratagene). Rat PP5 in pET21a was a gift from S. Rossie (Purdue University, West Lafayette, IN). pcDNA4B hCry1 and hCry2 were previously described (48). mClock and mBmal1 in pBluescript vector (J. Takahashi, Northwestern University, Evanston, IL) were cloned into pSG5 with Flag tags. *hPer1* was amplified from

HEK293T RNA by RT-PCR and cloned into pcDNA4B. pCS hPer2 was a gift from L. Ptáček (University of California, San Francisco). Additional constructs eliminating the His tag in pcDNA4B CK1 $\epsilon$ , Cry2, and Per1 were generated with the QuikChange Kit. All constructs were verified by DNA sequencing.

**Cell Culture and Antibodies.** HEK293T cells were purchased from American Type Culture Collection, RGC-5 cells (25) were a gift from N. Agarwal (University of North Texas Health Science Center, Fort Worth), and the BJ cell lines (33) were a gift from X.-F. Wang (Duke University, Durham, NC). HEK293T and RGC-5 cells were cultured in DMEM plus 10% FBS, and BJ cells were cultured in DMEM plus 20% FBS, under standard culture conditions. The following antibodies were used: Flag (Sigma); PP5 (BD Transduction Laboratories); myc (9E10), CKI $\epsilon$  (H-60), and PP1 (FL-18) (Santa Cruz Biotechnology); PER1, PER2, and BMAL1 (C. Lee, Florida State University, Tallahassee); and CRY1 (raised against a C-terminal peptide of mCRY1, NSNGNGGLMGYAP-GENVPSC). WBs were scanned and quantified by densitometry using IMAGEQUANT 5.0 software (Molecular Dynamics).

**Immunoprecipitation and Circadian Synchronization.** For coimmunoprecipitation, cells were lysed in Nonidet P-40 buffer (50 mM Tris, pH 7.5/150 mM NaCl/1 mM EDTA/0.5% Nonidet P-40) and incubated with immunoaffinity resins overnight at 4°C: HA

(Roche), microcystin (Upstate Biotechnology), protein A (Invitrogen), Flag M2 (Sigma), or His (Santa Cruz Biotechnology). Resin was washed three times with Nonidet P-40 buffer and eluted with SDS/PAGE sample buffer. For serum-induced cycling, BJ cells were grown to confluence, treated with DMEM plus 50% horse serum for 2 h (time = 0 at initiation of serum shock), and maintained in DMEM plus 0.5% FBS until harvest.

**Mouse Extract Preparation and *in Situ* Hybridization.** WT and mutant mice (49) housed under a 12:12 h light/dark schedule were killed at indicated ZTs ( $n = 3$ ; ZT0 = lights on). Livers were excised, minced, and ground in Nonidet P-40 buffer with protease inhibitors; extracts were clarified by centrifugation for 20 min at  $9,000 \times g$  at  $4^{\circ}\text{C}$ , and protein concentration was measured by Bradford assay. For *in situ* hybridization of brain sections, antisense and sense *PP5* probes were generated by *in vitro* transcription from pBluescript SK+ vector containing nucleotides 846–1481 of *mPP5* with T7 or T3 RNA polymerase, respectively (Promega), in the presence of [ $^{35}\text{S}$ ]-UTP. The *Per2* probe was generated, and *in situ* hybridizations were performed as described (50). *PP5* expression at ZT8 and ZT20 ( $n = 3$ ) was analyzed by one sample *t* test ( $P = 0.5$ ).

**Protein Expression and Purification.** Purification of CKI $\epsilon$  (14) and PP5 (29) from *Escherichia coli* and CRY2 (48) from Sf21 cells was performed as described. Myc-PER2 was purified from transiently transfected HEK293T cells with anti-myc (9E10) monoclonal antibody prebound to protein A/G agarose and washed stringently to reduce copurifying proteins. Each protein was analyzed for purity

by SDS/PAGE and silver stain, and concentration was estimated by comparison to known protein standards.

***In Vitro* Kinase and Phosphatase Assays.** Autophosphorylation of CKI $\epsilon$  (CKI $\epsilon$ -P) was done in kinase buffer [25 mM Tris, pH 7.5/10 mM MgCl $_2$ /0.1 mM ATP/1  $\mu\text{Ci}$  (1 Ci = 37 GBq) of [ $\gamma$ - $^{32}\text{P}$ ]ATP per reaction (3,000 Ci/mmol, NEN Research Products)] for 30 min at  $30^{\circ}\text{C}$ . To monitor dephosphorylation, 500 nM CKI $\epsilon$ -P was added to 100 nM PP5 in kinase buffer (all reactions with PP5 had 10  $\mu\text{M}$  palmitoyl-CoA, 2 mM MnCl $_2$ , and 1 mM iodoacetamide). Signals were obtained on PhosphorImager screens (Molecular Dynamics) and quantified by using IMAGEQUANT 5.0 software. Stimulation of CKI $\epsilon$  activity was measured by adding CKI $\epsilon$ -P to either PP5 or PP5 storage buffer (20 mM Tris, pH 7.6/4 mM MnCl $_2$ /0.1% 2-mercaptoethanol/50% glycerol) in the presence of 1  $\mu\text{g}$  of dephosphorylated casein (Sigma) or 500 ng of PER2-conjugated resin. Kinetic analyses of CRY2 inhibition were performed for 5 min at  $30^{\circ}\text{C}$ ; time course experiments showed the assay was linear for up to 15 min. CKI $\epsilon$  dephosphorylation was determined by normalizing  $^{32}\text{P}$  signal remaining after phosphatase treatment to the  $^{32}\text{P}$  signal intensity of CKI $\epsilon$ -P before treatment.

**Data Analysis.** All experiments were performed three or more times. Graphs are presented as means  $\pm$  standard error.

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